

Internalisation potential of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* and *Staphylococcus aureus* in lettuce seedlings and mature plants

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ABSTRACT

The internalisation potential of *Listeria monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* O157:H7 and *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* in lettuce was evaluated using seedlings grown in vermiculite in seedling trays as well as hydroponically grown lettuce. Sterile distilled water was spiked with one of the four human pathogenic bacteria (10^5 CFU/mL) and used to irrigate the plants. The potential for pathogen internalisation was investigated over time using light microscopy, transmission electron microscopy and viable plate counts. Additionally, the identities of the pathogens isolated from internal lettuce plant tissues were confirmed using polymerase chain reaction with pathogen-specific oligonucleotides. Internalisation of each of the human pathogens was evident in both lettuce seedlings and hydroponically grown mature lettuce plants. To our knowledge, this is the first report of *S. aureus* internalisation in lettuce plants. In addition, the levels of background microflora in the lettuce plants were determined by plate counting and the isolates identified using matrix-assisted laser ionisation–time of flight (MALDI–TOF). Background microflora assessments confirmed the absence of the four pathogens evaluated in this study. A low titre of previously described endophytes and soil inhabitants, i.e., *Enterobacter cloacae*, *Enterococcus faecalis*, *Lysinibacillus fusiformis*, *Rhodococcus rhodochrous*, *Staphylococcus epidermidis* and *Staphylococcus hominis* were identified.

Key words | foodborne human pathogens, internalisation

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INTRODUCTION

Dietary guidelines from governmental agencies and nationally recognised health professional organisations in many countries recommend an increase in the consumption of fresh fruit and vegetables (Beuchat 2002). Contrary to food products derived from animals, which are generally cooked, fruit and vegetables are mostly consumed raw. Fresh produce retains much of its natural microflora, including a diversity of bacteria, yeasts and fungi, of which, some may cause spoilage. Most of these natural microfloras represent epiphytic organisms with only a small number being plant pathogens (Beuchat 2002; Lindow & Brandl 2003). Human pathogens including *Escherichia coli* O157:H7,

Listeria monocytogenes, *Salmonella Typhimurium* and *Staphylococcus aureus* have been found associated with fresh produce (Takeuchi *et al.* 2000; Harris *et al.* 2003; Sharma *et al.* 2009). Rosenblueth & Martinez-Romero (2006) further demonstrated the endophytic nature of these organisms using ribotyping. It was reported in several papers that these pathogens are able to adapt and survive in the plant environment thereby introducing a potential food safety risk for the consumer (Deering *et al.* 2011a).

An increase in foodborne disease outbreaks associated with the increased consumption of contaminated fresh produce has been linked with a number of pathogenic bacteria

such as *E. coli* O157:H7, *Salmonella* spp. and *L. monocytogenes* (Johnston *et al.* 2006; Herman *et al.* 2008). From 1996 to 2005, the incidence of foodborne disease outbreaks linked to the consumption of leafy greens increased by 39% in the United States, while consumption of leafy greens increased by only 9% (Herman *et al.* 2008). In 2006, the consumption of *E. coli* O157:H7 contaminated fresh spinach caused 204 people to become ill in 26 states in the United States, three died and 31 cases progressed to haemolytic uraemic syndrome which often causes kidney failure (<http://www.cdc.gov/ecoli/2006/December/121406.htm>). In 2011, a multistate outbreak of *E. coli* O157:H7 in 10 states of the United States caused 60 individuals to become ill (<http://www.cdc.gov/ecoli/2011/ecoliO157/romainelettuce/120711/index.html>). A foodborne disease outbreak in the European Union in May 2011 was caused by *E. coli* O104:H4, a more virulent verocytotoxin-producing strain than *E. coli* O157:H7. The outbreak was eventually linked to a German sprout producer after first implicating Spanish cucumbers and tomatoes. The resultant €225 million losses per week of Spanish vegetable producers highlighted the economic impact of these outbreaks and the importance of accurate diagnostic test methods (<http://www.bbc.co.uk/news/world-europe-13683270>). The recent Listeriosis outbreak during 2011 was reported to be due to consumption of contaminated canteloupes that left 146 people sick and caused 30 deaths in 28 states across the United States (<http://www.foodsafetynews.com/2011/10/cantaloupe-listeria-outbreak-84-sick-15-dead>).

Contamination points during production and processing of fresh produce, that include irrigation water, soil, improper handling and storage practices, have been well studied (Guo *et al.* 2001; Brandl 2006). Most *E. coli* O157:H7 outbreaks can be linked directly or indirectly to faecal contamination of water and/or soil from cattle in integrated farming systems and/or humans through poor sanitation practices (National Advisory Committee on Microbiological Criteria for Food 1999; Beuchat 2002). In order to prevent disease outbreaks, good agricultural practices have been developed and are required to be implemented and maintained by farmers and retailers prior to procurement of the product.

A number of studies have demonstrated foodborne pathogen attachment to and colonisation of fruit and vegetable surfaces (Collignon & Korsten 2010). Some of the

pathogens have been reported to enter plant tissue via the roots (Solomon *et al.* 2002; Jablason *et al.* 2005; Cooley *et al.* 2006), stems or flowers prior to fruit development (Guo *et al.* 2001; Milillo *et al.* 2008). Once these pathogens have gained access to the internal tissues of the plant, transverse and vertical movement is possible. Internalisation of foodborne pathogens is thus of particular concern since removal through washing and contact surface disinfectants is unlikely (Warriner *et al.* 2003; Brandl 2006; Kroupitski *et al.* 2009).

Internalisation studies to date have focused mainly on either *E. coli* or *S. Typhimurium* (Deering *et al.* 2011a). The objective of this study was to investigate the potential of both Gram-negative (*E. coli* O157:H7, *S. Typhimurium*) and Gram-positive (*S. aureus*, *L. monocytogenes*) human pathogenic bacteria to internalise lettuce seedlings and mature plants in one study under similar controlled conditions. The internalisation of the specified pathogens was demonstrated using light and transmission electron microscopy (TEM), and viable plate counts with polymerase chain reaction (PCR) confirmation of pathogen identity. The background microflora was also isolated and identified using matrix-assisted laser ionisation–time of flight (MALDI-TOF).

MATERIALS AND METHODS

Bacterial strain and inoculum preparation

American Type Culture Collection (ATCC; Manassas, VA, USA) cultures *E. coli* O157:H7 (ATCC 35150), *L. monocytogenes* (ATCC 19115), *S. Typhimurium* (ATCC 14028) and *S. aureus* subsp. *aureus* (ATCC 12600) were used in this study. All cultures were maintained as lyophilised stocks and stored at -70°C with subcultures prepared 24 hours prior to use on Standard 1 medium. All media were obtained from Merck, Johannesburg, South Africa, unless stated otherwise. A single colony of each respective bacterium was subsequently inoculated into Tryptone Soy Broth (TSB) and shake incubated at 200 rpm at 37°C for 18 hours to obtain a final concentration of 10^8 CFU/mL. The concentration was confirmed by dilution plating onto selective agar (MacConkey agar for *E. coli* O157:H7, Oxford

Listeria agar for *L. monocytogenes*, Xylose lysine deoxycholate agar for *S. Typhimurium* and Baird-Parker agar for *S. aureus*).

Lettuce seedling germination, cultivation and inoculation

Butterhead lettuce seeds (Kirchhoffs, Honeydew, South Africa) (132 in total: 24 seeds for each foodborne bacteria, 18 each set for an untreated control and for a crystal violet test to confirm uptake and translocation) were planted 0.5 cm deep into six separate seedling trays each containing sterilised autoclaved medium grade vermiculite (Hygrotech, Pretoria, South Africa). The seeds were germinated at 25° C in a dark growth chamber for 2 weeks and seedlings were watered daily with sterile distilled water and half strength nutrient solution (according to the manufacturer's recommendations) (Ocean Agriculture (PTY) Ltd, Muldersdrift, South Africa).

Following germination, 12 hour light cycles were introduced and four seedling trays were watered daily with sterile distilled water containing the full strength nutrient solution inoculated with the respective pathogens (10^5 CFU/mL). No pathogens were added to the fifth seedling tray, serving as the negative control. One gram of crystal violet (Merck) was added to 500 mL of sterile distilled water and used to water the sixth seedling tray. On days 3, 5, 7, 14, 21 and 28 after planting, three seedlings from each tray were harvested.

Mature lettuce germination, cultivation and inoculation

Butterhead lettuce seeds (Kirchhoffs) (106 in total: 19 seeds for each foodborne bacteria, 15 for control and 15 for crystal violet tests) were planted into seedling trays and germinated as described previously. Following germination, 12 hour light cycles were introduced and all seedling trays were watered daily with sterile distilled water containing full strength nutrient solution as before. No pathogens were added. Four weeks after planting, the seedlings were removed, rinsed with water to ensure removal of any excess and transplanted to the hydroponic system (University of Pretoria: Experimental Farm, Pretoria). Lettuce plants were grown in the hydroponic system at a

temperature range of 25–30° C in sterile, water-tight containers with lids to prevent the leaves from touching the water. The control experiment was set up and crystal violet dye added as described previously. Sterile distilled water containing full strength nutrient solution and the respective bacterial inoculum (10^5 CFU/mL) was used to water the experimental plants weekly. Before inoculation and 7, 14, 21 and 28 days after planting, three mature lettuce plants from each treatment were removed and the roots and leaves harvested.

Weighing of seedlings and mature plants

The harvested lettuce seedlings and mature plants were weighed prior to surface disinfection and microbiological analysis. Lettuce seedlings were weighed as whole plants which included the leaves, stems and roots. The mature lettuce plants were divided into roots and leaves by cutting the short stem at the base of the leaves. Roots and leaves were weighed separately.

Verification of uptake and distribution

Seedlings and mature lettuce plants treated with crystal violet were cut vertically for visual verification of dye entrance, water uptake and distribution.

Effectiveness of surface disinfection

Butterhead lettuces (*Lactuca sativa* cv. Nadine) (28 days old) were separated into leaves and roots. Twenty-four leaves and roots were divided into two sets each (Set A surface disinfected and Set B non-surface disinfected). Leaves and roots within each set were subdivided into four subsets for each of the selected pathogens. Surfaces of leaves and roots in both Set A and Set B were inoculated with 50 µL respective bacterium inoculums using 10^5 CFU/mL. Inoculation was followed by air-drying in the laminar flow hood for 5 min. Set A was subsequently surface disinfected by immersion into 80% (v/v) ethanol for 5 min, followed by immersion twice in sterile distilled water to remove residual ethanol and air-dried at room temperature in the laminar flow hood (Solomon *et al.* 2002). Set B was not surface

disinfected in order to determine the residual pathogen titre on the leaves and roots.

Ten grams of the individual plant parts were placed into sterile polyethylene bags with 90 mL quarter strength Ringer's solution and homogenised for 5 min at 260 rpm in a Stomacher[®] 400 (Seward, Lasec SA (PTY) Ltd, Johannesburg, SA). The liquid phase was aseptically removed and filtered through a 0.45 µm cellulose nitrate filter (Sartorius, Johannesburg, SA). Each filter was added to 9 mL TSB, mixed by vortexing, serially diluted and plated onto the selective and Standard 1 agar in order to determine total viable bacterial counts. The plates were incubated at 37° C for 24 hours, counts recorded and transformed to $\log_{10}(x + 1)$ CFU/g.

Microbiological analysis of seedlings

The lettuce seedlings and mature plants were surface disinfected as previously described. Seedlings inoculated with the respective bacterial pathogens and the control were chopped in a sterile Petri dish in 1 mL quarter strength Ringer's solution using a sterile scalpel. Each seedling sample was subsequently serially diluted and plated onto selective agar and Standard 1 agar as described previously. The plates were incubated at 37° C for 24 hours, counts recorded and transformed to $\log_{10}(x + 1)$ CFU/g.

Microbiological analysis of mature plants

Ten grams of both mature lettuce roots and leaves were weighed aseptically, separated and placed into sterile polyethylene bags together with 90 mL quarter strength Ringer's solution and homogenised for 5 min at 260 rpm in a Stomacher[®] 400. The liquid phase was removed and filtered through a 0.45 µm cellulose nitrate filter. The filter was placed in 9 mL TSB, serially diluted, plated and incubated as described previously. Colony counts were recorded and transformed to $\log_{10}(x + 1)$ CFU/g.

DNA extraction and PCR

In order to confirm the presence of *E. coli* O157:H7, *L. monocytogenes*, *S. Typhimurium* and *S. aureus* in both the lettuce seedlings and mature lettuce plants, PCR was

performed using DNA extracted from the samples collected. The 0.45 µm cellulose nitrate filters were inoculated into TSB as described previously. Bacterial cells were cultured aerobically for 48 hours at 37° C with agitation at 200 rpm. The DNA was extracted using the Triton-X method described by Wang & Slavik (2005) with some modifications. One millilitre of the respective bacterial cultures was centrifuged at $6,000 \times g$ for 5 min. The process was repeated if the pellet was too small. The pellets were washed three times using 1 mL sterile double distilled water and subsequently centrifuged at $16,000 \times g$ for 5 min. The pellets were resuspended in 50 µL 1% (v/v) Triton X-100 (Sigma, Johannesburg, SA), boiled for 10 min at 100° C, cooled on ice for 10 min and centrifuged at $16,000 \times g$ for 5 min. Three microlitres of Ribonuclease A (Roche, Johannesburg, SA) was added to the supernatant and incubated overnight at room temperature to remove RNA present. A 1.5 µL sample of the supernatant was used as a template for each PCR. Each 25 µL PCR reaction mixture contained 0.3 µL of BioTaq polymerase (5 U/µL), 1.5 µL MgCl₂ (50 mM), 0.75 µL dNTPs (10 mM of each), 2.5 µL NH₄ reaction buffer (10×) (all from Bioline, Celtic Molecular Diagnostics, Cape Town, SA), 1.75 µL Bovine Serum Albumin Acetylated (10 mg/mL) (Promega, Madison, WI, USA), 1.25 µL Dimethyl Sulphoxide (Saarchem, Merck) and 0.3 µL of each primer (Whitehead Scientific, Cape Town, SA). Primers used in this study are listed in Table 1. For control purposes, a PCR reaction mixture containing sterile double distilled water and all other reagents except template DNA was included. Thermocycling was performed using an Eppendorf Thermocycler (Merck) and the PCR conditions were as follows: 94° C for 2 min, followed by 37 cycles of 94° C for 30 s, 61° C for 45 s and 72° C for 1.5 min, with a final extension at 72° C for 7 min. PCR products were visualised following gel electrophoresis on 1% agarose gels.

Identification of natural internal microflora using MALDI-TOF

Purified bacterial cultures isolated from Standard 1 agar medium for both seedlings and mature lettuce plants were transferred in duplicate directly to the MALDI-TOF steel polished target plate (Bruker, Bremen, Germany) and

Table 1 | Primers used for the detection of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Salmonella enterica* subsp. *enterica* and *Staphylococcus aureus* in lettuce seedlings and mature lettuce plants

Primer	Specificity	Sequence 5'-3'	Size (bp)	Reference
UidAa (30 pmol)	<i>Escherichia coli</i> O157:H7	GCGAAACTGTGGAATTGGG	252	Cebula <i>et al.</i> (1995)
UidAb (30 pmol)	<i>Escherichia coli</i> O157:H7	CGCTTTTGACACCTTAACCC	252	Cebula <i>et al.</i> (1995)
LMFP (20 pmol)	<i>Listeria monocytogenes</i>	AGCTCTTAGCTCCATGAGTT	450	Thomas <i>et al.</i> (1991)
LMRP (20 pmol)	<i>Listeria monocytogenes</i>	TCGAGAATCGAGGTACTCAA	450	Thomas <i>et al.</i> (1991)
SLDF (50 pmol)	<i>Salmonella enterica</i> subsp. <i>enterica</i>	CCTGTGAATGCCCTGATGAT	787	S. Collignon, unpublished data
SLDR (50 pmol)	<i>Salmonella enterica</i> subsp. <i>enterica</i>	GGACACTTACGGGACTACTA	787	S. Collignon, unpublished data
SCN2F (30 pmol)	<i>Staphylococcus aureus</i>	TTGCATATGTATGGCAATTGTT	655	S. Collignon, unpublished data
SCN2R (30 pmol)	<i>Staphylococcus aureus</i>	AACGTATACATACCGTTAACAA	655	S. Collignon, unpublished data

overlaid with the α -cyano-4-hydroxycinnamic acid matrix (Bruker). The target plate was subsequently analysed using Bruker MicroFlex LT MALDI-TOF in conjunction with Bruker Biotyper Automation Software and library. The MALDI-TOF was calibrated prior to use with the bacterial standard supplied by Bruker. Duplicate score values (SV) were recorded; SV were used to determine the accuracy of identification. A SV of between 1.999 and 1.700 was used to identify the genus name of the organism, and a value of above 2.0 was used to determine the genus and probable species of an organism.

Light and transmission electron microscopy – lettuce seedlings and mature plants

The primary root tip, branch point of newly developed lateral roots and the stem of the lettuce seedlings aged 1–4 weeks old were selected for sectioning. The primary root tip, branch point within the primary root at a mature lateral root junction and the stem node were selected for mature lettuce roots aged 4–8 weeks. For sectioning of the mature lettuce leaves 4–8 weeks old, the veins present at the base, middle and top of the lettuce leaves were selected. Following sectioning, the samples were fixed in 0.075 M phosphate buffer (pH 7.4) (Merck) containing 2.5% (v/v) glutaraldehyde (Merck). The samples were rinsed three times in 0.075 M phosphate buffer and fixed for 1 hour in 0.5% (v/v) aqueous osmium tetroxide (Merck). Subsequently, the samples were rinsed three times in 0.075 M phosphate buffer, followed by dehydration in a gradient of ethanol (50%, 70%, 90%, 100%, 100%, 100% (v/v))

(Merck) at 10 min intervals. The samples were infiltrated with 30% (v/v) Quetol (SPI-Supplies, West Chester, PA, USA) in ethanol for 1 hour, 60% (v/v) Quetol for 1 hour and a further 100% (v/v) Quetol overnight. Fresh pure Quetol was used to polymerise the samples at 60° C for 48 hours.

For light microscopy (LM), monitor sections (0.5 μ m) were cut, stained in Toluidine blue (Merck) and mounted in immersion oil before being examined using the Nikon Optiphot Transmitted Light Microscope (Nikon, Japan). LM sections were selected randomly for viewing. Following positive identification of bacteria within these sections, samples were cut into ultra-thin sections with an ultra microtome for TEM. The ultra-thin sections were contrasted in 4% (v/v) aqueous uranyl acetate (Merck) for 10 min, followed by Reynolds' lead citrate (Merck) for 2 min and examined using the Jeol JEM-2100F Field Emission Electron Microscope (JEOL, Japan).

Pure bacterial cultures of the respective pathogens were prepared for morphological comparisons. A single pure colony of each respective bacterium was prepared and inoculated into TSB as described previously. The cells were cultured aerobically for 48 hours at 37° C with shaking at 200 rpm. One millilitre of each bacterial culture was centrifuged at 6,000 \times g for 2 min and the process was repeated until a large enough pellet was obtained. The pellets were prepared for light and TEM as described previously. The following modifications were included for the TEM, samples were centrifuged at 6,000 \times g for 2 min and resuspended between each step. Following the addition of fresh pure Quetol, the pellets were not resuspended but allowed to

polymerise at 60° C for 48 hours. Samples were cut, contrasted, selected and viewed as described previously.

Data analysis

The data collected were analysed by analysis of variance procedures and means were separated using the Duncan's multiple range test at the 5% level. Statistical analyses were done using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA). To assess whether the same internalisation pattern observed in the lettuce seedlings could be seen with mature lettuce plants, plate counts for both roots and leaves were combined to give an overall whole plant analysis.

RESULTS

Effectiveness of surface disinfection

After surface disinfection of lettuce leaves and roots, a reduction in total viable bacteria numbers from 6.36 to 0.34 log CFU/g per lettuce leaf and from 6.92 to 0.30 log CFU/g per lettuce root was observed. Overall, the surface sterilisation effectiveness was 95% for both the lettuce leaves and roots.

Verification of dye uptake and distribution

Crystal violet dye could only be seen within the roots of seedlings grown in seedling trays (Figure 1). However, the dye was observed in both the roots and stems of mature lettuce plants grown in the hydroponic system (Figure 1). The root system was darkly stained, the stems were lightly stained and the leaf internal issue did not stain at all (Figures 1(e) and 1(f)).

Microbiological analysis of lettuce seedlings

Internalisation of *E. coli* O157:H7 in lettuce seedlings occurred after 3 days of irrigation with artificially contaminated water and could be detected for the entire 28 day duration of the experiment, ranging from 1.48 to 4.53 log CFU/g (Table 2). Statistical analysis showed that the number of internalised *E. coli* O157:H7 increased significantly from day 3 to day 5, with no significant differences in bacterial numbers noted from day 7 to day 28 (Table 2).

L. monocytogenes was internalised in lettuce seedlings following 5 days of irrigation as opposed to *E. coli* O157:H7 which was detected on day 3 (Table 2). The highest number of internalised *L. monocytogenes* was determined

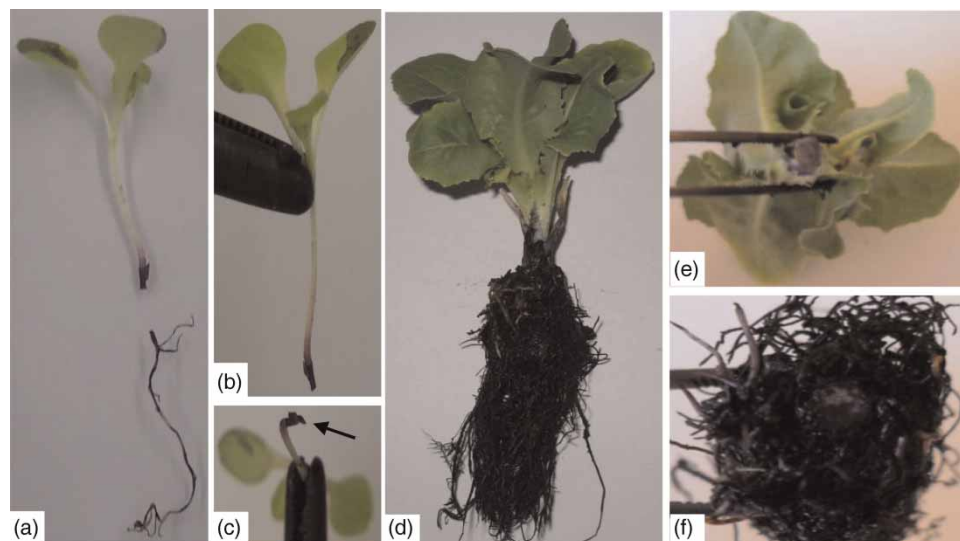


Figure 1 | Visual verification of crystal violet dye entrance and distribution in lettuce seedlings and mature plants. (a) Crystal violet dye present only in the root system of a 2-week-old lettuce seedling as indicated. (b) Absence of crystal violet dye within the stem of lettuce seedlings. (c) Cross-section through the lettuce seedling root system indicating the presence of crystal violet dye within the root system as indicated. (d) Crystal violet present in both the roots and stem of mature lettuce plants following 2 weeks' growth within a hydroponic system. (e) Cross-section showing the presence of crystal violet dye within the stem of mature lettuce plants. (f) Cross-section showing the presence of crystal violet dye within the root system of mature lettuce plants.

Table 2 | Average number of internalised human pathogenic bacteria in the lettuce seedlings over the 4-week detection period

Bacterial isolates	Log (CFU/g) of internalised foodborne bacteria					
	Day 3	Day 5	Day 7	Day 14	Day 21	Day 28
<i>Escherichia coli</i> O157:H7	1.48 ^{c*}	4.53 ^a	4.26 ^{ab}	1.94 ^{abc}	2.67 ^a	2.76 ^a
<i>Listeria monocytogenes</i>	ND	3.46 ^a	2.29 ^{ab}	1.17 ^{ab}	ND	ND
<i>Salmonella</i> Typhimurium	ND	ND	0.98 ^a	ND	1.14 ^a	1.35 ^a
<i>Staphylococcus aureus</i>	1.24 ^a	1.56 ^a	2.83 ^a	1.89 ^a	1.93 ^a	ND ^a

ND = none detected. No foodborne bacteria were found to be internalised.

The data collected were analysed by analysis of variance procedures, and means were separated using the Duncan's multiple range test at the 5% level. Statistical analyses were done using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA). *Means within rows followed by the same letters do not differ significantly ($P = 0.05$).

for day 5 (3.46 log CFU/g), whereafter it gradually decreased from day 7 to day 14, while the pathogen could not be isolated from the internal seedling tissue for the remainder of the experiment (Table 2). Comparison of bacterial numbers determined from day 3 to day 14 using statistical analysis showed that they were similar, and not significantly different (Table 2).

S. Typhimurium was internalised in lettuce seedlings only after 7 days of irrigation as opposed to *E. coli* O157:H7 and *L. monocytogenes* which were detected on day 3 and day 5, respectively. Statistical analysis showed that the bacterial numbers determined from day 7 to day 28, ranging from 0.98 to 1.35 log CFU/g were similar and not significantly different (Table 2).

S. aureus was isolated from lettuce seedlings from day 3 to day 21 at similar levels, not significantly different, ranging from 1.24 to 2.83 log CFU/g. On day 28, *S. aureus* was not detected following plating onto selective agar and the absence of the pathogen was confirmed by PCR analysis (Table 2).

The identities of all presumptive colonies isolated from the respective selective media were confirmed by PCR analysis (results not shown). Similarly, the absence of the four pathogens in the control lettuce seedlings was confirmed using PCR analysis.

Lettuce seedlings grown in seedling trays with sterile growth medium and irrigated with sterile water (negative control) still retained natural microflora. Natural microflora titres in the lettuce seedlings were present at non-significantly different levels from day 3 to day 28 (Table 3). Identification of the natural internal microflora isolates

Table 3 | Average number of natural microflora present in the control lettuce seedlings and mature lettuce roots, leaves and whole plants grown in seedling trays and a water culture hydroponic system, respectively, over a 4-week period

Controls	Log (CFU/g) of natural microflora					
	Day 3	Day 5	Day 7	Day 14	Day 21	Day 28
Lettuce seedlings	4.01 ^{a*}	5.19 ^{ab}	3.78 ^{ab}	4.25 ^{ab}	3.23 ^b	3.12 ^b
Mature lettuce roots	N/A	N/A	7.06 ^a	6.43 ^a	7.27 ^a	7.08 ^a
Mature lettuce leaves	N/A	N/A	5.92 ^{ab}	5.92 ^{ab}	5.88 ^{ab}	6.92 ^a
Whole mature plants	N/A	N/A	7.06 ^{ab}	6.60 ^b	7.29 ^{ab}	7.78 ^a

The data collected were analysed by analysis of variance procedures, and means were separated using the Duncan's multiple range test at the 5% level. Statistical analyses were done using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA).

*Means within rows followed by the same letters do not differ significantly ($P = 0.05$).

using the MALDI-TOF revealed the presence of several well-known bacterial spp. in the lettuce seedlings. These included *Enterobacter cloacae* (SV: 1.941 & 2.091), *Enterococcus faecalis* (SV: 2.388 & 2.398), *Lysinibacillus fusiformis* (SV: 1.803 & 1.957), *Rhodococcus rhodochrous* (SV: 2.068 & 2.027), *Staphylococcus epidermidis* (SV: 2.208 & 1.830) and *Staphylococcus hominis* (SV: 2.055 & 2.080). *Enterococcus faecalis* and *S. hominis* were most abundant in the seedlings, followed by *R. rhodochrous*, *E. cloacae*, *L. fusiformis* and *S. epidermidis*.

Comparison of the weight of the lettuce seedlings infected with the four respective pathogens to that of the control seedlings showed no real significant difference (results not shown).

Microbiological analysis of mature lettuce plants

Similar to lettuce seedlings, natural internal microflora was present in the control roots and leaves of mature lettuce plants grown in sterile water (Table 3). Natural internal microflora was detected in the mature lettuce plants for the entire duration of the experiment (Table 3). Analysis of the control roots indicated that natural microflora titres were found to be not significantly different, ranging from 6.43 to 7.27 log CFU/g (Table 3). The bacterial titres in the control leaves were the highest on day 28 (6.92 log CFU/g) and significantly different from day 7 with the lowest bacterial count (4.83 log CFU/g) measured on day 21 (Table 3). Bacterial titres for the whole plants ranged from 6.60 to 7.78 log CFU/g (Table 3).

The natural internal microflora isolated from roots and leaves of mature lettuce plants were identified as *Arthrobacter creatinolyticus* (SV: 2.101 & 2.319), *Enterobacter cloacae* (SV: 2.320 & 2.238), *Lysinibacillus fusiformis* (SV: 1.944 & 2.083) and *Microbacterium arborescens* (SV: 1.967 & 2.297). In addition, *Enterococcus faecalis* (SV: 2.411 & 2.354) was found to be present in the mature lettuce roots. *Microbacterium arborescens* was found to be the most abundant in the mature lettuce leaves followed by *A. creatinolyticus*, *L. fusiformis* and *E. cloacae*. In comparison, *E. faecalis* was found to be most abundant in the mature lettuce roots followed by *E. cloacae*, *L. fusiformis*, *M. arborescens* and *A. creatinolyticus*.

None of the four pathogens was found to be present in mature lettuce plants prior to inoculation and transplanting.

E. coli O157:H7 was found internalised in both the roots and leaves of mature lettuce plants in the hydroponic system after 7, 14, 21 and 28 days (Table 4). No significant difference in internalisation levels for this pathogen in the roots were observed throughout the entire 28 day period, with viable plate counts ranging from 4.98 to 6.37 log CFU/g (Table 4). A significant increase of internalised *E. coli* O157:H7 from day 7 (3.70 log CFU/g) to day 14 (5.17 log CFU/g) was found in the lettuce leaves, followed by no significant difference for the remainder of the experiment. The *E. coli* O157:H7 internalisation levels in the whole plants ranged from 5.01 log CFU/g on day 7 to 6.38 log CFU/g on day 21 (Table 4).

Internalisation levels of *L. monocytogenes* in the roots ranged from 2.97 to 5.75 log CFU/g over the 28 day period. The pathogen levels determined for days 7, 14 and 28 were similar and not significantly different (Table 4). *L. monocytogenes* titres fluctuated in the lettuce leaves from 2.26 log CFU/g (lowest) to 6.01 log CFU/g (Table 4). *L. monocytogenes* titres fluctuated in the whole plants, ranging from 4.33 log CFU/g on day 21 to 6.11 log CFU/g on day 7 (Table 4).

Internalisation levels of *S. Typhimurium* in the roots of mature lettuce plants were not significantly different from day 7 (4.16 log CFU/g) to day 21 (4.73 log CFU/g), however a significant increase was observed on day 28 (5.87 log CFU/g) (Table 4). Similar to *L. monocytogenes*, the detection levels of *S. Typhimurium* in the lettuce leaves were found to fluctuate over the 28 day period ranging from 0.84 to 3.79 log CFU/g and were not significantly different

Table 4 | Average number of internalised human pathogenic bacteria in mature lettuce roots, leaves and whole plants grown in a water culture hydroponic system over a 4-week period

Bacterial isolates	Log (CFU/g) of internalised bacteria											
	Day 7			Day 14			Day 21			Day 28		
	Roots	Leaves	Whole	Roots	Leaves	Whole	Roots	Leaves	Whole	Roots	Leaves	Whole
<i>Escherichia coli</i> O157:H7	4.98 ^{a*}	3.70 ^b	5.01 ^b	5.75 ^a	5.17 ^a	5.94 ^{ab}	6.71 ^a	4.39 ^b	6.38 ^a	3.79 ^a	4.35 ^b	5.18 ^{ab}
<i>Listeria monocytogenes</i>	5.21 ^{ab}	6.01 ^a	6.11 ^a	2.97 ^b	4.77 ^{ab}	4.89 ^b	4.29 ^{ab}	2.26 ^c	4.33 ^b	5.75 ^a	2.95 ^{bc}	5.75 ^a
<i>Salmonella</i> Typhimurium	4.16 ^b	2.68 ^a	4.34 ^a	4.16 ^b	3.79 ^a	4.39 ^a	4.73 ^b	0.84 ^a	4.73 ^a	5.87 ^a	3.78 ^a	5.89 ^a
<i>Staphylococcus aureus</i>	4.05 ^b	4.63 ^b	4.79 ^{bc}	5.15 ^b	5.87 ^a	5.96 ^{ab}	4.48 ^b	3.82 ^c	4.64 ^c	7.12 ^a	4.25 ^{bc}	7.1 ^a

Roots, leaves and whole plants were analysed separately. The data collected were analysed by analysis of variance procedures, and means were separated using the Duncan's multiple range test at the 5% level. Statistical analyses were done using SAS 9.2 software (SAS Institute Inc., Cary, NC, USA).

*Means within rows followed by the same letters do not differ significantly ($P = 0.05$).

(Table 4). Whole plant analysis revealed a continuous gradual increase in internalised levels of *S. Typhimurium* from day 7 (4.34 log CFU/g) to day 28 (5.89 log CFU/g). Statistical analysis showed that the internalisation levels were similar and not significantly different (Table 4).

The internalisation levels of *S. aureus* in lettuce roots were not significantly different from day 7 (4.06 log CFU/g) to day 21 (4.48 log CFU/g), however a significant increase was observed on day 28 (7.1 log CFU/g), which is a similar pattern to that observed for *S. Typhimurium* in the mature lettuce roots (Table 4). The levels of internalised pathogen in the lettuce leaves increased significantly from day 7 (4.63 log CFU/g) to day 14 (5.87 log CFU/g), whereafter a significant decrease was observed on day 21 (3.82 log CFU/g) (Table 4). Bacterial titres found for the whole plants ranged from 4.64 to 5.96 log CFU/g from day 7 to day 21, with a significant increase to 7.1 log CFU/g found on day 28 (Table 4).

Comparison of the weight of both the roots and leaves of the pathogen inoculated mature lettuce plants to that of the control mature lettuce plants showed no real significant differences.

All presumptive pathogen isolates detected in mature plants by plate counts on the respective selective media were confirmed by PCR analysis using pathogen-specific primers. The absence of the four human pathogenic bacteria in the mature control plants was confirmed by PCR analysis of the microflora isolated from the plants.

Light and transmission electron microscopy

Pure cultures of the respective pathogens were used as a reference point when viewed under the LM in order to confirm the presence of the pathogens in the lettuce root and leaf cell (Figure 2). Long rod-shaped bacteria were present in both mature lettuce leaves and roots inoculated with *E. coli* O157:H7 (Figure 2(a)); short rods for *L. monocytogenes* (Figure 2(b)) and *S. Typhimurium* (Figure 2(c)); and cocci-shaped bacteria for *S. aureus* (Figure 2(d)).

TEM revealed long rod-shaped bacteria (3.20 µm in length) in mature lettuce leaves and roots contaminated with *E. coli* O157:H7 (Figure 3(a)). Short rod-shaped bacteria (1.25 µm in length) could be observed in mature lettuce leaf and root cells contaminated with *L.*

monocytogenes (Figure 3(b)). Furthermore, short rods averaging 1.63 µm were observed in the cells of mature lettuce leaves and roots contaminated with *S. Typhimurium* (Figure 3(c)). Lastly, cocci-shaped bacteria averaging 1 µm were observed in the cells of the mature lettuce leaves and roots contaminated with *S. aureus* (Figure 3(d)).

To confirm that the rod- and cocci-shaped structures observed in the mature lettuce leaves and roots were indeed bacteria, the images were magnified to confirm the presence of a double cellular bacterial cell membrane (images not shown). Cell shape and size of the waterborne pathogens were confirmed using the control pathogens prepared. The structures observed in the contaminated lettuce leaves and roots were similar and characteristic to those observed in the control micrographs (Figure 3). In addition to the presence of bacterial cells, the cells could be seen to be dividing and thus multiplying inside the lettuce plant, as seen in Figure 3.

DISCUSSION

This study clearly demonstrated the internalisation potential of *E. coli* O157:H7, *S. Typhimurium*, *L. monocytogenes* and *S. aureus* in lettuce seedlings and mature hydroponically grown lettuce plants. Although the *Staphylococcus* genera are commonly encountered in the endophytic population of plants, the ability of the *S. aureus* human pathogen to internalise in plants has, to our knowledge, not been reported to date. Sixty and seventy per cent, respectively, of studies summarised in a review paper by Deering *et al.* (2011a) reported the internalisation of *E. coli* O157:H7 and *Salmonella* spp. in fresh produce. In contrast, 40 and 30% of studies respectively reported that they found no evidence of internalisation of these pathogens. In our internalisation study, sterile distilled water was spiked with either one of the four human pathogenic bacteria (10⁵ CFU/mL) and used to irrigate the lettuce seedlings grown in vermiculite and mature lettuce plants grown in a hydroponic system. Similar to our study, hydroponic systems were also used in other studies to investigate the internalisation potential of human pathogens in tomatoes (Guo *et al.* 2001), spinach (Warriner *et al.* 2003), maize (Bernstein *et al.* 2007) and lettuce (Franz *et al.* 2007). Other internalisation studies used

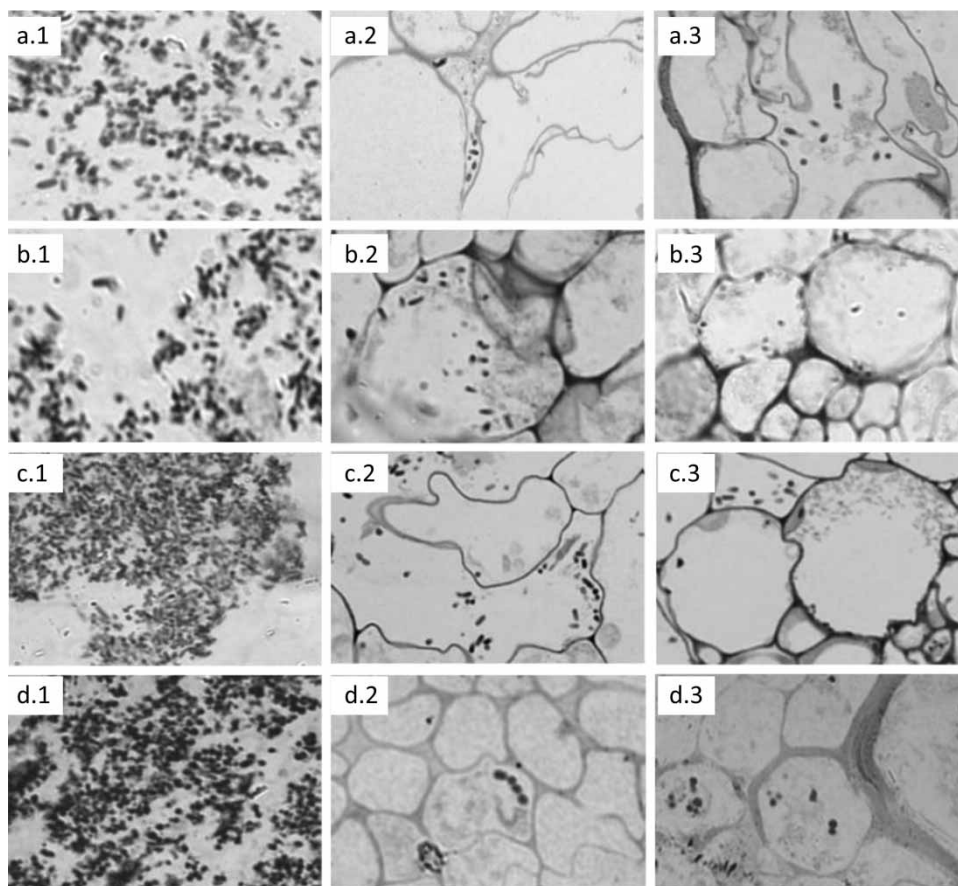


Figure 2 | Light microscopy of foodborne pathogens used to artificially inoculate water and found to internalise in the cells of mature leaves and roots viewed under 100× oil immersion objective. (a) *Escherichia coli* O157:H7; (b) *Listeria monocytogenes*; (c) *Salmonella enterica* subsp. *enteric* serovar *Typhimurium*; (d) *Staphylococcus aureus*. (1) Liquid culture control, (2) presence in mature leaves and (3) presence in mature roots.

soil (Warriner *et al.* 2003; Bernstein *et al.* 2007; Mootian *et al.* 2009; Sharma *et al.* 2009; Erickson *et al.* 2010a) or compost (Erickson *et al.* 2010c) as growth medium, or directly inoculated leaf surfaces (Zhang *et al.* 2009) or seed (Jablasone *et al.* 2005; Miles *et al.* 2009).

Methods used to date to show internalisation of *E. coli* O157:H7 and *Salmonella* spp. within plants included the following: plating onto appropriate growth media, LM, scanning electron microscopy (SEM), TEM, immunolocalisation, fluorescence *in situ* hybridisation (FISH) and immunoblot tissue printing (Deering *et al.* 2011a). In this study, methods selected to confirm internalisation included enumeration of viable bacteria by plating onto selective growth media, PCR confirmation of pathogen identity, LM as well as TEM. To verify the presence of the pathogens, a representative colony for each of the test organisms was

included in the LM and TEM study to compare bacterial cell wall structure and cell size. In order to verify the absence of the four human pathogens in uninoculated lettuce leaves the natural viable background microflora was determined and the isolates identified using MALDI-TOF analysis. It is clear from published data that each method used to investigate potential internalisation of microbes has its limitations, however using a combination of methods supports the findings in this study.

Concentrations ranging from as high as 10^9 CFU/mL of *E. coli* O157:H7 and *S. Typhimurium* (Franz *et al.* 2007) to as low as 10^2 CFU/mL of *E. coli* O157:H7 and *S. Typhimurium* (Jablasone *et al.* 2005) have been used to inoculate lettuce seeds in previous studies. Erickson *et al.* (2010b) reported no internalisation when lettuce leaves were inoculated with 10^6 log CFU/mL (4.4 log CFU per leaf) *E. coli*

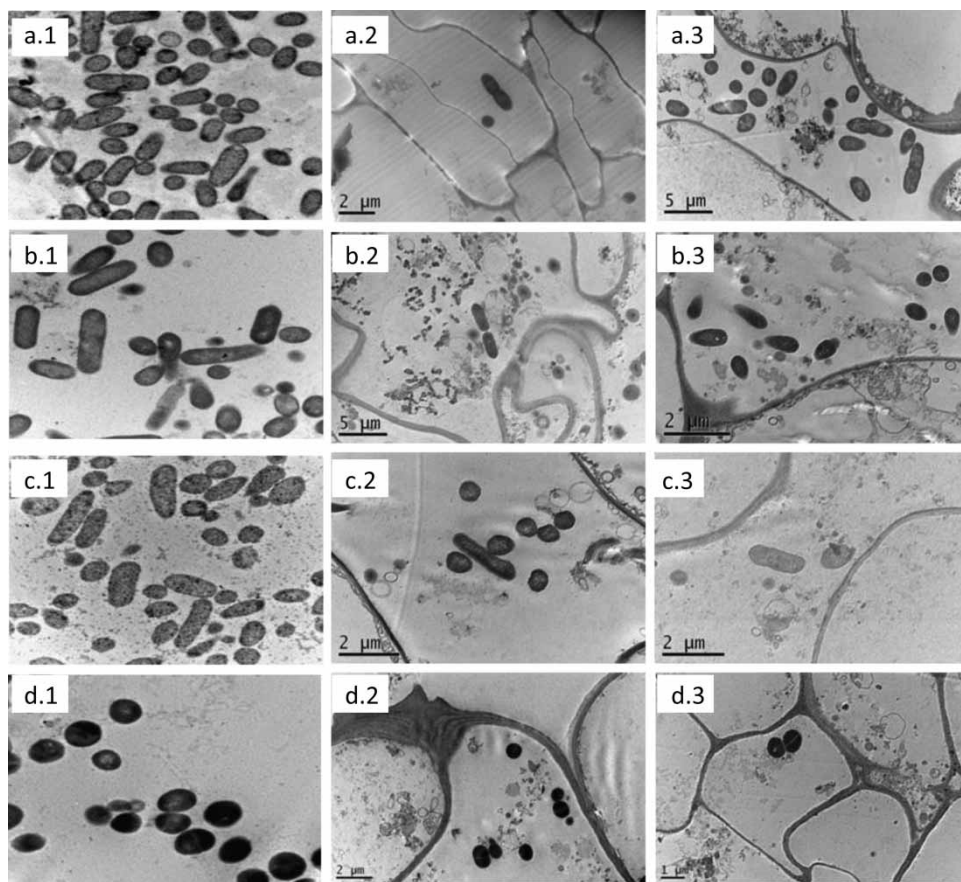


Figure 3 | Transmission electron micrographs of foodborne pathogenic bacteria used to artificially inoculate water and found to internalise inter- and intra-cellularly in mature leaves and roots. (a) *Escherichia coli* O157:H7; (b) *Listeria monocytogenes*; (c) *Salmonella enterica* subsp. *enterica* serovar *Typhimurium*; (d) *Staphylococcus aureus*. (1) Liquid culture control, (2) presence in mature leaves and (3) presence in mature roots.

O157:H7, but it did occur when inoculated with 10^8 CFU/mL (6.4 log per leaf). The inoculation concentration used in this study was 10^5 CFU/mL. An interesting observation was that even though initial inoculation concentrations differ between studies, the final concentration of internalised organisms was similar. In the present study, internalisation levels of *E. coli* O157:H7 remained relatively constant, reaching a high of 4.53 log CFU/g, suggesting the seedlings had reached a maximum carrying capacity (Table 2). This is consistent with the study by Franz *et al.* (2007) where a similar carrying capacity of 3.95 (± 1.02) log CFU/g of *E. coli* O157:H7 in lettuce seedlings was recorded. In a recent study by Erickson *et al.* (2010b), 2.9 (± 1.1) log CFU/g internalised *E. coli* O157:H7 was recorded for lettuce leaves, which is similar to previously reported internalisation levels.

Two major routes of human pathogenic bacteria colonisation and internalisation have been reported to date: (1) entrance through natural plant openings such as stomata, lenticels, sites of lateral root emergence and/or biological or physically damaged sites; (2) following irrigation of plants, soaking of seeds or washing of fresh produce during processing after harvest (Solomon *et al.* 2002; Jablonsone *et al.* 2005; Cooley *et al.* 2006; Deering *et al.* 2011a). In the case of the latter, bacteria are pulled along with the movement of water as the water enters internal plant tissues. The four human pathogens tested in this study entered the plants through water uptake, as was evident due to the tracking of crystal violet dye uptake in the roots and stems.

Several mechanisms of human pathogen internalisation in plants have been postulated and are extensively studied at the moment (Kroupitski *et al.* 2009). The first major obstacle

for entry of human pathogenic bacteria into plant tissues is the rigid and chemically complex host cell wall (Aparna *et al.* 2009; Deering *et al.* 2011b). The ability of plant pathogens to produce enzymes such as cellulases, xylanases, pectinases and proteases that break down cell walls, has been reported to provide a point of entry for the pathogen (Aparna *et al.* 2009; Deering *et al.* 2011a). *Salmonella* is a member of the *Enterobacteriaceae* family that includes the soft rot *Erwiniae*, which produce cell wall degrading enzymes. A cellulase able to degrade cellulosic substrates has been isolated from *S. Typhimurium* (Yoo *et al.* 2004). Additionally, *Salmonella* isolated from infected *Arabidopsis* plants were equally virulent for human cells and mice, which indicates the *Salmonella* infection mechanisms for plants and animals are similar (Schikora *et al.* 2011). *Salmonella* spp. induce phagocytosis in humans and once internalised, destroy the cell.

In contrast to the *Salmonella* infection mechanism, human pathogenesis studies showed that *E. coli* O157:H7 did not destroy the host cell by phagocytosis. *E. coli* O157:H7 adhere to the extracellular regions of the host cell, ultimately causing damage to the cell structure through complex processes, resulting in inflammation and the inability of the cell to absorb nutrients, thereby enabling the pathogen to enter the host cell (Deering *et al.* 2011b).

There are no reports in the literature of *S. aureus* cellulosic degrading enzyme isolation and characterisation. However, a putative endo-1,4 beta-glucanase has recently been identified for *S. aureus* RF122 (EMBL Bank AJ938182). Interestingly, mutants of *S. aureus* strain with human pathogenesis genes inactivated, were attenuated in their ability to infect *A. thaliana*. These results indicate that as was observed for *Salmonella*, *S. aureus* infection mechanisms for plants and animals might be similar. Although *S. aureus* has not been implicated in foodborne disease outbreaks, comparison to infection mechanisms in humans could facilitate the elucidation of internalisation mechanisms of Gram-positive bacteria in plants.

Using *L. monocytogenes* expressing green fluorescent protein (GFP), the pathogen was visualised in the intercellular spaces of *A. thaliana* leaves, suggesting internalisation through stomata (Milillo *et al.* 2008). The internalisation of *L. monocytogenes* in the lettuce plants observed during this study is of concern, since it may pose a health risk.

The recent Listeriosis outbreak due to contaminated cantaloupe consumption in the USA emphasises the importance of the findings in this study (<http://www.foodsafetynews.com/2011/10/cantaloupe-listeria-outbreak-84-sick-15-dead>).

Future work should focus on the possibility of contamination under field conditions. Enhanced knowledge of the comparative behaviour of human bacterial pathogens and both epiphytic and endophytic bacteria through co-inoculation, as well as plant-microbe interactions will provide valuable information on both plant and bacterial factors involved in internalisation. Further work is also needed to better understand the mechanisms involved in the ability of *S. aureus* and *L. monocytogenes* to survive as endophytes in fresh produce and migrate through plant cell tissues. This should be greatly facilitated by the large amount of genomic and proteomic data now available to shed light on the role of numerous genes that still have unknown functions. Some of these genes may be important in crop plants grown in nature and therefore may provide clues to developing new methods to detect contamination of products with human bacterial pathogens.

CONCLUSIONS

In this study, the potential of human pathogenic bacteria (Gram-positive and Gram-negative) to internalise lettuce seedlings grown in vermiculite in seedling trays and hydroponically grown mature plants in a pre-harvest environment, was confirmed. Regardless of the modes of infiltration, internal tissues of the edible portions of fresh produce can provide a microenvironment for bacteria where they are protected from removal, allowing colonisation and potential survival. Improved monitoring and water quality control is therefore needed to avoid fresh produce contamination. This study highlights the potential impact of using contaminated agricultural water in fresh produce production and processing systems on food safety. Although the controlled environment used to study potential internalisation does not mimic the complexity of the natural environment, it does show the potential of endophytic presence of human pathogens in plant tissue and provides additional support for similar internalisation studies.

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